In-vitro replication of Chelonid herpesvirus 5 in organotypic skin cultures from Hawaiian green turtles (Chelonia mydas)

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Abstract: Fibropapillomatosis (FP) is a tumor disease of marine turtles associated with Chelonid herpesvirus 5 (ChHV5) that has historically been refractory to growth in tissue culture. Here, we show for the first time de novo formation of ChHV5-positive intranuclear inclusions in cultured green turtle cells, which is indicative for active lytic replication of the virus. The minimal requirements to achieve lytic replication in cultured cells included 1) either in-vitro culturing of ChHV5-positive tumor biopsies (plugs) or organotypic cultures (rafts) consisting of ChHV5-positive turtle fibroblasts in collagen rafts seeded with turtle keratinocytes and 2) keratinocyte maturation induced by raising raft or biopsy cultures to the air-liquid interface. Virus growth was confirmed by detailed electron microscopic studies revealing intranuclear sun-shaped capsid factories, tubules, various stages of capsid formation, nuclear export by budding into the perinuclear space, tegumentation, and envelopment to complete de novo virus production. Membrane synthesis was also observed as a sign for active viral replication. Interestingly, cytoplasmic particles became associated with keratin filaments, a feature not seen in conventional monolayer cell cultures where most studies of herpesvirus replication have been performed. Our findings draw a rich and realistic picture of ChHV5 replication in cells derived from its natural host and may be crucial not only to better understand ChHV5 circulation but also to eventually complete Koch's postulates for FP. Moreover, the principles described here may serve as model to culture other viruses that are resistant to replication in conventional cell culture.Importance: A major challenge in virology is viruses that cannot be grown in the laboratory. One example is Chelonid herpesvirus 5 (ChHV5) associated with fibropapillomatosis, a globally distributed, debilitating, and fatal tumor disease of endangered marine turtles. Pathology shows that ChHV5 is shed in skin. Here, we show that ChHV5 will grow in vitro if we replicate the complex three- dimensional structure of turtle skin. Moreover, lytic virus growth requires a close interplay between fibroblasts and keratinocytes. Finally, morphogenesis of herpesviral growth in three dimensional cultures reveals a far richer, and likely more realistic, array of capsid morphologies than that encountered in traditional monolayer cell cultures. Our findings have application to other viruses including those of humans.

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In-vitro replication of Chelonid herpesvirus 5 in organotypic skin cultures from Hawaiian green turtles (*Chelonia mydas*)

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Abstract: Fibropapillomatosis (FP) is a tumor disease of marine turtles associated with Chelonid herpesvirus 5 (ChHV5) that has historically been refractory to growth in tissue culture. Here, we show for the first time *de novo* formation of ChHV5-positive intranuclear inclusions in cultured green turtle cells, which is indicative for active lytic replication of the virus. The minimal requirements to achieve lytic replication in cultured cells included 1) either *in-vitro* culturing of ChHV5-positive tumor biopsies (plugs) or organotypic cultures (rafts) consisting of ChHV5-positive turtle fibroblasts in collagen rafts seeded with turtle keratinocytes and 2) keratinocyte maturation induced by raising raft or biopsy cultures to the air-liquid interface. Virus growth was confirmed by detailed electron microscopic studies revealing intranuclear sun-shaped capsid factories, tubules, various stages of capsid formation, nuclear export by budding into the perinuclear space, tegumentation, and envelopment to complete *de novo* virus production. Membrane synthesis was also observed as a sign for active viral replication. Interestingly, cytoplasmic particles became associated with keratin filaments, a feature not seen in conventional monolayer cell cultures where most studies of herpesvirus replication have been performed. Our findings draw a rich and realistic picture of ChHV5 replication in cells derived from its natural host and may be crucial not only to better understand ChHV5 circulation but also to eventually complete Koch's postulates for FP. Moreover, the principles described here may serve as model to culture other viruses that are resistant to replication in conventional cell culture.

Key words: Herpesvirus, morphogenesis, electron microscopy, immunohistochemistry, pathology

Importance: A major challenge in virology is viruses that cannot be grown in the laboratory. One example is Chelonid herpesvirus 5 (ChHV5) associated with fibropapillomatosis, a globally distributed, debilitating, and fatal tumor disease of endangered marine turtles. Pathology shows that ChHV5 is shed in skin. Here, we show that ChHV5 will grow *in vitro* if we replicate the complex three- dimensional

structure of turtle skin. Moreover, lytic virus growth requires a close interplay between fibroblasts and keratinocytes. Finally, morphogenesis of herpesviral growth in three dimensional cultures reveals a far richer, and likely more realistic, array of capsid morphologies than that encountered in traditional monolayer cell cultures. Our findings have application to other viruses including those of humans.

Introduction

Seven species of sea turtles exist globally with the most common being the green turtle (*Chelonia mydas*) (58). Green turtles are listed as threatened or endangered throughout their range by the US Endangered Species Act and International Union for the Conservation of Nature; threats include loss of nesting habitat, nest depredation, and bycatch in commercial fisheries (19). Diseases have also been documented in green turtles the most common of which is fibropapillomatosis (FP) that has a global distribution (27) and causes disfiguring tumors on the skin, eyes and mouth and internal tumors, mostly in the lungs, heart, and kidneys (29, 76). FP has been seen in most places where green turtles range, and has been most extensively studied in Hawaii, Florida, Brazil, and Australia (27, 38). Severely tumored turtles become immunosuppressed, septic, emaciated, and eventually die (77, 81). In Hawaii, FP continues to be a leading cause of debilitation and stranding of green turtles although the disease there is waning for unknown reasons (11). In contrast, in Florida (21) and Brazil (6), prevalence of FP remains relatively constant.

FP is likely caused by an infectious agent. Experimentally, FP is transmissible in green turtles by cell free-filtrates (31), and the disease appears contagious in captive turtles (36). Although numerous etiologies and cofactors have been proposed for FP including parasites (17), marine toxins (43), pollutants (2), or habitat eutrophication (65), the most likely cause is a herpesvirus. On light microscopy, epidermal intranuclear inclusions (EII) have been seen in tumors that on electron microscopy contain herpesvirus-like particles (35). Additionally, herpesviral DNA is consistently

detected in tumored but less commonly in non-tumored tissues in turtles from Hawaii (61), Florida (41), and Brazil (64). In 2012, the virus associated with FP, Chelonid herpesvirus 5 (ChHV5), was designated as the type for the newly erected genus Scutavirus of the subfamily alphaherpesvirinae, family Herpesviridae (1).

A big limitation to our understanding of the pathogenesis and epidemiology of FP in green turtles is the inability to grow ChHV5 *in-vitro*. In nature, lytic viral production infrequently occurs in keratinocytes (KC) of skin tumors (78). Extensive attempts to grow the virus using cultured normal or tumor fibroblasts (FB) or KC monolayers from green turtles in the presence or absence of compounds that induce herpesvirus growth *in-vitro* have failed. Apparently, the virus persists in a predominantly latent state, and infectivity is lost as cells are passaged (79). In this context, ChHV5 resembles certain other herpesviruses, such as human herpesvirus 8, that are difficult to culture *in-vitro* (62) and that contrast with more easily cultured herpesviruses such as Herpes simplex (HSV) (74). Inability to culture ChHV5 has stymied attempts to fulfill Koch's postulates and to develop serological tests thereby limiting our understanding of the epidemiology of FP or potential routes of transmission.

ChHV5 appears more akin to papillomaviruses (49) in requiring stratified KC to undergo lytic replication. As such, organotypic cultures might provide an alternate means of culturing ChHV5 by recapitulating the three-dimensional structure of turtle skin including dermis, basement membrane, and strata basale, spinosum, granulosum, and corneum (23). Organotypic cultures were first developed in the late 1970s by Green and colleagues who constructed rafts of collagen embedded with mouse FB and overlaid with cultured KC that were raised to the air-liquid interface, thereby stimulating stratification of KC from stratum basale to corneum (25). This system then allowed *in-vitro* cultivation of papillomaviruses that heretofore had been refractory to cell culture (49).

This study sheds new insights in herpesviral replication. Here, we show that ChHV5 will grow in green turtle skin biopsies (plugs) and organotypic cultures (rafts). Our findings are the first instance of

using this method in a non-mammalian organism and of culturing ChHV5 *in-vitro*. Although rafts have been developed for other mammals such as rats (59), dogs (68), and horses (10), we know of no such system for Reptilia (birds and reptiles). Ability to maintain tumor explants in-vitro could be a tool to understand broader questions in tumor cell biology, wound repair, and epitheliotropic herpesviral reproduction in reptiles.

Results

Origin of tissues

We obtained plugs from 90 tumors and 10 sections of normal skin (neck) from 24 turtles sampling 1-7 (Mean=4) tumors/case. Tumors originated mostly from front flippers (51%), neck (19%), rear flippers (14%), jaw hinge (10%), cloaca, eye, glottis (1% each), and unrecorded locations (remainder). Of the 90 tumors sampled, we performed histological examination on 52 and EII were detected in 9 (17%) tumors. Forty-five of the 52 tumors assessed histologically were measured and had surface area ranging from 3-10,600 cm^2 (mean±SD= 297±1228 cm^2). There was no significant relationship between tumor size and presence of EII; however, the statistical power to detect this by logistic regression given our sample size and variability of the tumor measurements was low (p = 0.0491).

Light microscopy cultured plugs

We examined 1073 cultured plugs by histology of which 176 originated from normal skin and the remainder from tumors. Of 176 cultured plugs from normal skin, 139 had intact epidermis, and none had EII. Of 892 cultured plugs from tumors, 753 had intact epidermis, and of these, 87 (11.5%) had locally extensive ballooning degeneration of epidermis with EII exclusively in the epidermis (Fig. 1a,b). In areas of ballooning degeneration, green turtles mounted an immune response to EII and cell membranes as measured by reactivity against turtle 7S IgY using monoclonal anti-turtle IgY antibodies (Fig. 1c) whereas anti-ChHV5 VP26 rat polyclonal antibody decorated mainly nuclei and cell membranes

faintly (Fig. 1d). Negative controls (FP positive serum against normal skin, FP negative serum against inclusions, and FP Positive serum with Campylobacter mab) failed to decorate inclusions.

Of the 753 cultured plugs with intact epidermis, 693 came from tumors with measurements, and these were used to statistically analyze culture conditions conducive to formation of EII. Of the factors analyzed (tumor size, days plugs equilibrated submerged, days plugs cultured at air-liquid interface, or use of inducers), none contributed significantly to formation of EII (Table 1). To assess whether culturing tumors influenced EII production, we examined histology in separate samples of 52 tumors prior to and after culture. While only 17 % (9 of 52) of tumors had EII, the detection rate of EII in tissues from the same tumors after culture increased to 42% (22 of 52), a difference that was statistically significant (Chi^2= 7.8; p=0.0095; OR= 3.5; 95% CI=1.4 to 8.1) thereby confirming that culturing tissues increased virus production. Of 33 tumors from which EII positive plugs were cultured, 21 originated from front flippers, six from the neck, two each from the rear flipper and mouth, and one each from the cloaca and eye.

Light microscopy rafts

For rafts, FB originated from 27 turtles (21 tumors and 11 normal skins) whereas KC originated from 13 cases (14 tumors and 8 normal skins). Of 87 rafts attempted, 69 developed an intact stratified epidermis with a stratum corneum, and of these, eight (11%) presented ballooning degeneration and EII exclusively in epidermis (Fig. 1e, f). Compared to plugs, there was more uniform reactivity to 7S IgY of FP positive turtles (Fig. 1g) whereas anti-VP26 polyclonal antibody reacted mainly with nuclei (Fig. 1h). Negative control tissues failed to show reactivity to inclusions. When tumor status (+/-) of FB or KC were examined for their influence on formation of EII in rafts, there were four combinations: FB+KC+, FB+KC-, FB-KC+, FB-KC-. Proportions of attempts for each of these categories yielding EII in rafts were as follows: FB+KC+ (7/30; 23%); FB+KC- (1/19; 5%); FB-KC+ (0/14; 0%); FB-/KC- (0/16; 0%). Interestingly, EII were seen only in rafts using early passage tumor fibroblasts known to be positive for viral DNA (79). It

appeared that tumored KC were more permissive to lytic viral growth and formation of EII than non-tumored, although neither of them contain viral DNA when cultured in the absence of tumor FB (79). Moreover, of the culture parameters analyzed by logistic regression, only days collagen raft incubated prior to addition of KC contributed significantly to formation of EII, and alpha value was marginally lower than 0.05 (Table 2). These observations clearly emphasize the need for cell culture and the requirement for the organotypic arrangement, with intimate contact between tumored FB and KC that greatly enhanced the generation of EII.

Electron microscopy-Nuclear morphology

Electron microscopy of normal turtle skin revealed basement membrane, strata basale (Fig. 2a), spinosum, granulosum, and corneum with no ballooning degeneration or virus-associated structures in cytoplasm or nucleus. Compared to healthy cells from non-tumored skin, cells from plug cultures had histologic evidence of ballooning degeneration (Fig. 2b) with markedly vacuolated cytoplasm and enlarged swollen nuclei with marginated chromatin and ill-defined nuclear membranes. Within the nucleus, aggregates of capsids were sometimes seen within or around dense or reticulated nuclear bodies. Dense bodies were round, ranged from 927-2671 (1706±508, N=15) (Mean±SD, N) nm diameter (Fig. 2c), and were surrounded by a unilayered corona of capsids (Fig. 2d) looking similar to intranuclear sun-like structures described previously in the herpesvirus literature (5, 40, 46, 53, 57). Tangential sections of these bodies suggested them to be uniformly covered with or consisting of capsids (Fig. 2e). Reticulated bodies were more amorphous, ranged from 786-1823 (1128±420, N=6) nm at their widest point, and had variably sized irregular internal cavities populated by capsids some of which looked to be in the process of being assembled (Fig. 2f, Fig. 3a). Occasional tubular arrays of crystalline material were seen in the nucleus associated with capsids. These tubules, with an outer diameter of ca. 80 nm, appeared to segment and constrict at the ends eventually forming spheres (Fig. 3b-c). Apparent reduplication of nuclear membranes was evident (Fig. 3d). Virus-infected nuclei from plugs contained

five distinct morphologies of viral capsids. Stippled scaffold capsids (Fig. 4a; Fig. 5a) were oblong with irregularly arranged internal dots, clear capsids (Fig. 4b; Fig. 5a) had no internal structures, , targetoid scaffold capsids (Fig. 4c; Fig. 5a) were clear with a round or linear central electron-dense core , concentric capsids (Fig. 4d; Fig. 2f) enclosed a circular scaffold, and solid capsids (Fig. 4f; Fig. 5b) had a uniform electron-dense appearance. Of 971 capsids enumerated in 19 nuclei, 734 (75.6%) were stippled followed by 123 (12.7%) clear, 95 (9.8%) concentric, 16 (1.6%) targetoid, and 3 (0.3%) were solid. There was no significant difference in diameters for all five capsid types (Table 3). Of 44 plug cells examined on electron microscopy, seven (16%) had dense bodies, eight (18%) had reticulated bodies, nine (20%) had crystalline arrays, and six (14%) had apparent reduplication of nuclear membrane.

Cell pathology of rafts was broadly similar to that of plugs. Of 647 nuclear capsids enumerated in 18 nuclei, 529 (82%) were concentric (Fig. 5c), 91 (14%) were clear, 17 (3%) were solid, and the remainder were targetoid; stippled capsids were absent. Reticulated bodies with partially formed capsids and tubules were present (Fig. 5d), round dense bodies were absent, and capsids within cytoplasm were rare. Capsids within the nucleus in rafts were about the same size as those in plugs (Table 3). Of 20 cells enumerated in rafts, four had reticulated bodies and six had tubules within the nucleus.

Electron microscopy-Cytoplasmic morphology

Capsids were rarely seen transiting between the nucleus and cytoplasm by budding (Fig. 5e) whereupon they appeared to accumulate near the nucleus and became associated with keratin filaments (Fig. 5f). Within plug cytoplasm, three types of round-to-elliptical viral particles were seen including non-tegumented or tegumented (Fig. 6a), and enveloped encompassing single (Fig. 6b) and multiple (Fig. 6c) capsids. Non-tegumented and tegumented capsids were associated with electron-dense keratin filaments whereas enveloped capsids were more often associated with vesicles and delicate fibrils. Electron-dense material with capsids appeared to sequester at edges of affected cells

(Fig. 6d). Of 1003 particles enumerated in the cytoplasm of 51 cells of plugs, tegumented were most common (587 or 58.5%) followed by non-tegumented (301 or 30%), with enveloped particles comprising the remainder. Cytoplasmic capsids were twice as large as those in the nucleus, and there was no significant difference in mean diameter between capsid morphologies within cytoplasm (Table 3). Within rafts, cytoplasmic capsids were infrequently encountered but were similar to those of plugs.

Discussion

Growth of ChHV5 requires intimate contact between fibroblasts and keratinocytes

The presence of a herpesvirus associated with FP has been known for over 20 years (35), yet the virus has been refractory to in-vitro culture (79). This is the first time organotypic skin or plug cultures have been developed for a non-mammalian host allowing lytic growth of ChHV5 confirmed by immunohistochemistry and electron microscopy. Two lines of evidence suggest we were actually replicating virus *de novo* rather than merely maintaining existing virus in culture. First, for plugs, the greater than 3 fold increase in odds of obtaining EII in tissues from tumors after culture argues strongly that we were generating EII *in-vitro*. Second, for rafts, the presence of stratified KC with ballooning degeneration and EII further supported that viral replication was occurring *de novo*, because turtle KC in monoculture do not develop a stratified morphology with stratum corneum, nor do they support lytic growth of ChHV5, nor are they positive for herpesviral DNA by qPCR (79).

Our work with rafts suggests that ChHV5 production requires a close interplay between tumored FB and maturing KC. Ballooning degeneration and EII were only seen in the epidermis of plugs or only after the rafts had been raised to the air-liquid interface thereby stimulating maturation of KC (25) and pointing to the critical role that this cell type plays in allowing completion of the replicative cycle of ChHV5. Amplification of viral infectivity in the extreme periphery (maturing KC) makes biological sense, because this represents the major source of virus transmission (78). The important role of tumored FB is illustrated in part by raft cultures where KC from normal skin were able to support the *de novo*

formation of virus replication sites, although only when they had been layered on top of tumored FB. The obligate requirement of tumored FB, that have been documented to be consistently qPCR positive for herpesviral DNA (79), for production of EII in rafts suggests this cell type plays an important role in virus growth in KC. Exploring how cell signaling between FB and KC influences virus morphogenesis may be a promising avenue of research. Perhaps FB may represent an important site of latency for ChHV5. Although we failed to detect virus by electron microscopy in fibroblasts, the virus may reactivate at low abundance before being transferred to the KC. This hypothesis fits with the reactivation of latent HSV1 or HSV2 as a consequence of co-cultivating hardly permissive, latently infected neurons with susceptible and permissive cell lines, such as Vero cells (4, 72).

Interestingly, the use of chemical inducers to grow herpesviruses refractory to *in-vitro* cultivation did not appear to positively influence production of lytic virus growth in rafts or plugs. This also confirms previous attempts to culture ChHV5 in turtle FB or KC where inducers failed to increase viral loads in latently infected monolayers of turtle fibroblasts as tested by qPCR (79) However, we caution that given the low rate of EII production in this study and the variety of inducers tried, it is likely that our ability to detect a statistically significant effect was underpowered. Future studies might benefit from being more strategic in selection of inducers and focusing on fewer chemicals.

Unique morphologic features of ChHV5

For plugs, the most striking findings were highly regular round electron-dense bodies surrounded by viral capsids. Intranuclear amorphous dense bodies associated with variable numbers of viral capsids have been seen in monolayer cell cultures infected with pseudorabies (PRV) (40), cytomegalovirus (CMV) (57), and HSV1 (60). While viral proteins were identified in nuclear dense bodies of cells infected with HSV (5) and PRV (40), dense bodies were devoid of nucleic acids (8). Our study is the first to show dense bodies with such a uniform geometry and capsid coverage. Reticulated nuclear bodies with an electron-dense fibrillar network enclosing mainly concentric capsids and partial capsids

also does not seem to be documented in the literature; the closest we could find to this is Figure 14 in CMV infected cells (69). Similar to the same study, we also observed spherical and polyhedral forms of capsids, which represent different forms of capsid maturation. Finally, we encountered seemingly partial capsids in intranuclear reticular bodies (Fig. 3a) which were remarkably similar to late partial HSV1 capsid assembly in a cell-free system (51). The close association of capsids with these electron-dense bodies, along with evidence of viral proteins in other studies (40, 57), suggests they are capsid factories. Alternatively, these bodies are dumping grounds for malformed capsids, but having a virus devote resources to create such large amounts of non-viable particles would seem to make little evolutionary sense from a replication efficiency standpoint.

The variety of capsids seen in plugs was also unusual. Clear, concentric, and targetoid capsids were similar to type A, B and C capsids described for HSV (63); however we most often saw capsids with stippled scaffolds most similar to those described in CMV (69) or varicella zoster (52) and electron dense capsids filled with DNA. For Marek's disease (MDV), type A (clear capsids) are most common with type B (concentric) considered abortive and type C are mature (18); however clear criteria justifying why type B were abortive are lacking. For CMV (73) and PRV (48), type B is most common and is considered an intermediate form between A and C. In contrast, stippled capsids were most common in ChHV5-infected plugs with types A and C forming a minority. The close association of stippled and concentric capsids with dense bodies (capsid factories) suggests them to be immature, perhaps transitioning from the second most numerous clear capsids, to targetoid, then solid forms. Answering this definitively will require more controlled temporal observations of plugs over time, something not feasible given the low (10%) success rate of viral replication we are achieving currently.

Intranuclear tubular structures associated with partial capsid formation were similar to those observed for lymphocytes infected with human herpesvirus 6 (55), HSV2-infected vero cells (54), MDV-infected lymphocytes (9), CMV-infected FB, and at least 8 other herpesviruses (3). These tubules have

been proposed, though without providing supportive data, to represent abortive capsids that somehow happen to differentiate into tubular structures due to mistakes in capsid assembly (3, 9). While this may have its logic in the context of infections at exorbitantly high multiplicity of infection (MOI), it would seem maladaptive for viruses in the context of natural replication at low MOI to commit host cell resources to construct such large abortive structures. An equally plausible, though somewhat provocative explanation, may be that these tubular structures represent an early form of capsid production where capsids are being assembled via tubular intermediates. Until data are provided focusing on morphogenesis of these tubules along with identification of viral proteins, their role in herpesviral replication remains an unexplained but a potentially important mechanism of viral capsid assembly. Such investigations for ChHV5 await the development of reagents that would allow us to tag viral proteins for electron microscopy.

Intermediate filaments play an important role in development of ChHV5

There was a clear association of naked and tegumented intracytoplasmic capsids with keratin filaments in plugs, and reasons for this remain unknown. Intermediate filaments, of which keratins are a component, are synthesized from cell periphery to center and play an important role in herpesvirus infection facilitating transport of virus across cell membranes (32). Keratins may also inhibit migration of inflammatory cells to the epidermis thus shielding viruses from the host immune response (22). Supporting this conjecture is pathology of epidermal fibropapillomas in green turtles where dermal lymphoid infiltrates are common whereas epidermal inflammation is rare (30). Thus, maturing KC may offer the only refugia where the virus can carry out the entire lytic phase of reproduction and may protect virus when skin cells are shed thereby facilitating transmission. Similarities exist with other herpesviruses such as Epstein Barr (EBV) where virus remains latent in lymphocytes but sheds in mucosal epithelium (75).

In contrast to other herpesviridae where tegumentation and envelopment is associated with Golgi complex (47), tegumented particles of ChHV5 appeared more closely associated with keratin filaments whereas enveloped particles occurred in electron-dense material in the cytoplasm, often involving multiple capsids. Capsids in electron-dense material has been seen in MDV (16), Lucke (71), and CMV where, in the latter case, the dense material contains virus tegument proteins (24). In ChHV5, this electron-dense material containing capsids segregated to cell surfaces suggesting tropism to cell membranes or junctions thereby providing a potential mechanism of cell-cell movement of virus. A parallel exists for HSV1 where mature virus has a tropism for desmosomes, tight, and adherent junctions (37).

The need to examine herpesviral development in a 3D matrix

Organotypic cultures have been used to look at development of HSV (33), HCMV (26), and EBV (75), but none examined virus morphogenesis at the ultrastructural level. Indeed, most studies of herpesvirus morphogenesis that focus on electron microscopy have looked at viral growth in monolayer cell cultures. Virus morphogenesis varies depending on the cell culture system (67). Moreover, in many instances, virus isolates were first adapted for optimal growth in cell cultures before electron microscopy studies were carried out. Given this, it is highly doubtful that what is seen in monolayers with cell culture adapted virus strains is completely reflective of what happens in the more complex three-dimensional environment of host tissues with wild type virus. This point is illustrated when we compare ChHV5 virus morphogenesis in cultured plug biopsies from tumors that most closely replicate the host environment versus rafts where KC were seeded on a rat tail collagen matrix containing turtle FB. Keratinocytes from plugs had 5 morphologies of intranuclear capsids (clear, concentric, targetoid, solid, stippled) with stippled morphology dominating, presence of nuclear round bodies surrounded with capsids, and numerous intracytoplasmic capsids associated with keratin filaments. Contrast this with raft KC where concentric capsids dominated in the nucleus, stippled capsids and intranuclear round

bodies were absent, and intracytoplasmic capsids were infrequent. Given that plugs are relatively easier to culture than rafts and yield a richer picture of virus morphogenesis, future studies might focus on optimizing plug cultures.

Whilst the rafts and plug culture technique are promising ways to grow ChHV5 *in-vitro*, we acknowledge several limitations. Screening for EII in tumors relied on light microscopy examination of a single 5 µm thick slice of tissue from each tumor. Given the rarity of EII in tumors (78), it is highly likely we missed many tumors with EII. In contrast, given their small size (3-6 mm), lesions in plugs occupied a comparatively higher proportion of the tissue compared to tumors thereby facilitating more detailed observations of virus growth. Finally, our inability to culture turtle KC through numerous passages and lack of access to persistently infected FB cell lines required a constant source of tumors and cells which, for a threatened species like green turtles, could limit the widespread applicability of using rafts or plugs to grow virus. Techniques exist to immortalize cells such as manipulating telomerase (42) or use of stem cells, and these might be future leads to follow. For instance, avian KC stem cells were successfully used to grow MDV (15), and similar techniques might be applicable to turtles.

We think it improbable that our findings are part of the normal morphology of turtle cells because none of the structures seen in rafts or plugs were present in normal skin of turtles. It is also unlikely that the structures seen here were artefacts of viral inducers because all the electron micrographs shown here were from cultures that received no viral inducers. Our findings thus paint a picture of ChHV5 morphogenesis that adopts features of various herpesviruses and provides potential new insights into herpesviral capsid assembly. Three-dimensional cultures generate a richer variety and morphology of viral structures than are typically seen in monolayers, and host tissues yield greater diversity of forms than rafts thus confirming studies of monolayers where different cell types yield different morphologies (67). Future studies of human herpesvirus morphogenesis might benefit from closer scrutiny of virus development in tissues that more closely replicate the three-dimensional

structure of the host. Finally, our findings may help towards management of FP in wild turtles. A better understanding of virus morphogenesis coupled with further refinement of this system may lead ways to grow cell-free ChHV5 in monolayers. This in turn would allow confirmation the virus as a cause of FP, validation of serological tests to help elucidate epidemiology and transmission of ChHV5, all critical pieces of information to aid future management and mitigation of this disease in endangered green turtles.

Materials and Methods

Tissue samples originated from green turtles with severe cases of FP that had a poor prognosis and merited euthanasia for humane reasons (76). Turtles originated from the islands of Kauai, Oahu, Maui and Hawaii between 2007 and 2009. Immediately after euthanasia, skin biopsy sites were extensively scrubbed with soap and water with copious rinsing to remove epibionts (algae; barnacles) for 5 min. Subsequently, skin was scrubbed with betadine surgical scrub for 5 min, excised with sterile forceps and scalpel, placed in phosphate buffered saline containing 200 U/ml penicillin, 0.2 mg/ml streptomycin sulfate, 10 µg/ml gentamicin sulfate, and 5 µg/ml amphotericin B (Sigma-Aldrich), and maintained at 4 C until processing within 12 h of collection. Skin from normal wild or captive healthy turtles was prepared the same way except that sampling was done with 6 mm biopsy punches after infusion of 2% lidocaine at the biopsy site. A subset of tumors was measured and surface area estimated as described (78). We used two strategies to grow ChHV5 in turtle KC, incubation of plugs and rafts.

Tissue plugs

Skin collected from necropsy or biopsies was washed twice in sterile PBS, 3 or 6-mm plugs were collected using sterile disposable skin biopsy punches (Acuderm), trimmed with sterile scalpel, and 2-4 punches per well of a 24-well plate were equilibrated in Leibowitz L-15 (44) supplemented with 10% fetal calf serum (FCS, Hyclone), 1mM sodium butyrate (Sigma), 1mM non-essential amino acids

(Hyclone), and growth factors (KGM-2 Single Quots, Lonza) (KC media) at 30C for 3-7 d. Equilibrated tissue plugs were elevated to the air-liquid interface by placing onto mesh grids in 6-well plates containing 3 ml of KC media and incubated for 4-29 d.

Some plugs were treated with the following chemicals known to induce lytic growth of herpesviruses in tissue culture: 0.5 (n=21) and 1mM (n=31) sodium butyrate or 8 (n=127) and 16nM (n=54) phorbol 12-myristate 13-acetate (TPA) for 24 h every 4 d (82), 10 µM 5-azacytidine(n=6) (13) or 50 µM forskolin (n=29) for 4 d (70), 5 mM hexamethylenebisacetamide (HMBA; n=8) daily (7), 10ng/ml tumor necrosis factor alpha (TNF-a-n=8) daily (39), 20 µg /ml iododeoxyuridine (IUDR-n=11) for 48 h (50), 10nM dexamethasone (n=2) for 48h or IUDR for 24h followed by 10nM dexamethasone (n=3) for 72h (56), 1nM epinephrine (n=5) for 24h (12), and 5 µM trichostatin A (n=9) or 10mM valproic acid (n=4) for 18h (14). All chemicals were from Sigma, except for dexamethasone and epinephrine, which were from Phoenix Pharmaceutical and MP Biomedicals, respectively. After air-liquid interface cultivation, tissue plugs were harvested, half fixed in 10% neutral buffered formalin for standard histopathology examination and the other half either frozen (-70C) or saved in electron microscopy fixative (45).

Rafts (organotypic cultures)

For rafts, green turtle KC and FB were cultured in KC media (as above) and FB media (Leibovitz L-15, 10% FCS, 1mM sodium butyrate (Sigma), 1mM non-essential amino acids (Hyclone), 100 U/ml penicillin, 0.1mg/ml streptomycin sulfate, 5 µg/ml gentamicin sulfate and 2.5 µg/ml amphotericin B, all from Sigma), respectively. Fibroblasts and KC were identified based on morphology and reactivity to vimentin and pancytokeratin (79). Fibroblasts from tumors were initiated from primary culture or once frozen stock at passage 3 or less to ensure that cells were virus positive by qPCR (79) whereas FB from normal tissue were initiated from primary culture or once frozen stock (passages 1-43). Collagen gels were made of two parts of 5X L-15, one part reconstitution buffer (2.2% $NaHCO_3$, 0.05M NaOH, and

0.2M HEPES buffer, filter sterilized using 0.22 µm), 6.5 parts of rat tail collagen type 1 in 0.02N acetic acid (BD) at concentrations ranging from 2 to 6.1 mg/ml, and 0.5 part of cell suspension at concentrations ranging from 1-5x10^5 to 2x10^6 cells/gel. Fibroblasts and collagen were mixed, 0.5-2.5 ml added to 12 or 24-well plates, and allowed to polymerize at 37C for 1 h. Gels were then equilibrated in FB media for 0-7 d at 30C. Keratinocytes were cultured in KC media and when at 80-90% confluence, cells were harvested and overlaid on rafts at concentrations ranging from 1.5-4.75 x 10^5 cells/gel, and cultured submerged at 30C for 1-13 d. Afterwards, cultures were mechanically detached by sliding a sterile spatula around the gel and incubated for 1 h to 4 d at 30 or 37C in order for the cultures to shrink and become elastic. Cultures were then raised to the air-liquid interface and incubated for an additional 10-30 d. Postculture, rafts were processed as for plugs.

Immunohistochemistry (IHC)

For histopathology, rafts or plugs were embedded in paraffin, sectioned at 5 um, stained with hematoxylin and eosin (H&E), and examined for presence of characteristic EII indicative of herpesviral infection (78). To confirm whether EII were ChHV5, we reacted slides with anti-ChHV5 polyclonal rat antibody (78). We also confirmed that EII could be recognized as foreign antigens by green turtles as follows: skin tumors with EII detected with H&E were deparaffinized, rehydrated in ethanol, equilibrated in EnVision Flex wash buffer (Dako), and drained. Antigen was recovered with treatment by proteinase K (Dako) for 5 min and washed. Non-specific peroxidase was blocked with 3% hydrogen peroxide for 10 min followed by washing. Tissues were blocked with 5% non-fat milk in borate buffer pH 8 (MB) containing 0.5M NaCl (high salt) for 30 min, drained, incubated for 30 min with FP+ turtle serum diluted 1:50 in high salt MB, washed, incubated 30 min with anti-7S IgY monoclonal antibodies (80) at 2 µg/ml in antibody diluent (Dako), washed, incubated 30 min with ImmPRESS peroxidase Universal goat anti-rabbit/ mouse neat (Vector Labs), washed twice, and reaction visualized with diamino benzidine (Dako). To ensure specificity of turtle antibodies to EII, we ran the following controls: FP negative serum against

tissues with EII and FP positive serum against normal skin. To ensure specificity of reactivity of anti-7S mabs, we also ran FP positive serum against turtle skin with EII but used a mab against Campylobacter instead of anti-7S mabs. Negative control for VP26 were run as described (78).

Electron microscopy

For electron microscopy, tissues were rinsed in 0.1 M sodium cacodylate buffer containing 0.35M sucrose and post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer. Tissue was dehydrated in a graded ethanol series, substituted with propylene oxide, and embedded in LX112 epoxy resin. Epoxy embedded tissues were cut into 1 µm thick sections and stained with Richardson's for light microscopy. For electron microscopy, ultrathin (60-80 nm) sections were obtained on an RMC Powertome ultramicrotome, double stained with uranyl acetate and lead citrate, viewed on a Hitachi HT7700 TEM at 100 kV, and photographed with an AMT XR-41B 2k x 2k CCD camera. We enumerated morphologic types of capsids in the nucleus and cytoplasm. All virus-associated structures were measured using Image J (66). Electron microscopy of normal turtle skin served as a morphologic control.

Statistics

To assess how culture conditions or tumor size influenced presence/absence of EII, we used Firth's logistic regression (20) with R package logistf (28). This form of logistic regression uses a penalized maximum likelihood to account for complete or quasi separation of the outcome variable (presence/absence of EII) for data sets with low (<20%) percentages of 1 or 0 outcomes. To assess whether cultured plugs with EII were more or less likely to originate from tumors with EII as assessed by histology, we used chi square analysis. Sizes of viral capsids were compared with standard analysis of variance. All statistical analyses were done with R (34) with alpha=0.05.

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631 **Figure 1. Light microscopy of plugs (a-d) and rafts (e-h); bar =** 100 µm for (a) and (e) and 50 µm for all
632 other panels. *Morphologic highlights*: Note stratified epidermis (a,e) with ballooning degeneration
633 (arrows) and somewhat less cellular collagen matrix for raft (e) compared to denser more cellular dermis
634 with plug (a). Note EII (arrows) in epidermis of plugs (b) and rafts (f). Turtle 7S IgY reactivity assayed by
635 monoclonal antibodies recognizes EII and cell membranes in plugs (c) whereas staining for raft
636 epidermis is more intense (g). In contrast, anti-VP26 polyclonal recognizes mainly nuclei in plugs (d) and
637 rafts (h). *Culture conditions*: Plug (a-d) incubated submerged 4 d, air-liquid interface 17 d. Raft (e, h)
638 tumor fibroblast gel incubated submerged 7 d, tumored KC added and incubated submerged 6 d, air-
639 liquid interface 11 d. Raft (f) tumor FB gel incubated submerged 1 d, tumored KC added and incubated
640 submerged 2d, air-liquid interface 13 d. Raft (g) tumor FB gel seeded with tumored KC, cultured
641 submerged 7d, air-liquid interface 13 d.

642 **Figure 2. Normal skin and plug nuclei electron micrographs.** a) Healthy keratinocyte (stratum basale)
643 from normal skin; note basement membrane (black arrowhead), mitochondria (black arrow), keratin
644 filaments (white arrowhead), and indistinct cell borders delineated by desmosomes (white arrow); bar =
645 1 µm. b) Keratinocyte infected with ChHV5; note enlarged swollen nucleus with marginated chromatin
646 (black arrow), numerous capsids (white arrow), round dense body surrounded by capsids (white
647 arrowhead) the whole of which is surrounded by markedly vacuolated cytoplasm; bar = 1 µm. c) Dense
648 intranuclear body surrounded by uniform array of stippled capsids; bar = 200 nm. d) Detail of dense
649 intranuclear body; bar = 100 nm. e) Tangential section through dense intranuclear body; note capsids in
650 various planes of section; bar=200 nm. f) Reticulated intranuclear body replete with concentric capsids;
651 bar = 200 nm.

652 **Figure 3. Plug nuclei electron micrographs (continued)** a) Reticulated intranuclear body containing
653 concentric or stippled capsids. Note partially formed capsids (white arrows); bar = 200 nm. b)
654 Intranuclear crystalline tubular arrays; note segmentation of tubules (white arrow) apparently

progressing to constriction of ends of segment (black arrow) to circular form (arrowhead); bar= 200 nm. c) Intranuclear crystalline arrays; note clear (black arrow) and stippled (white arrow) capsids nearby and tubular fragments apparently in process of constricting at ends (arrowhead); bar = 200 nm. d) Apparent reduplication of nuclear membranes; bar = 500 nm.

Figure 4. Capsid morphology of ChHV5 seen in nucleus (a-f) or cytoplasm (f-h) of cultured rafts or plugs. a) Stippled (plugs only); b) Clear; c) Targetoid; d) Concentric-plug; e) Concentric-raft; f) Solid (cytoplasm and nucleus rafts and plugs); g) Tegumented-plugs; h) Enveloped-plugs. Sizes of capsids are in Table 3.

Figure 5. Plug nuclei electron micrographs (continued)(a,b); Nucleus of rafts (c, d); nuclear cytoplasmic transition of plugs (e-f). a) Reticulated intranuclear body with stippled (white arrow), clear (black arrow) and targetoid (white arrowheads) capsids; bar = 200 nm. b) Detail of nucleus with clear, stippled, and solid (white arrow) capsids; bar = 200 nm. c) Nucleus of raft culture with close up of commonly encountered intranuclear concentric capsids; bar=100 nm. d) Reticulated intranuclear body within raft culture associated with clear (black arrow), concentric (white arrow), and incomplete (black arrowhead) capsids along with tubular crystalline material (white arrowhead); bar = 200 nm; n=nucleus. e) Budding of viral particle (arrow); note the dense intrusion of inner nuclear membrane going into the perinuclear space; bar=200 nm. f) Perinuclear accumulation of dense capsids filled with DNA (black arrow) closely associated with keratin filaments (white arrow) and adjacent to fragmented nuclear membrane; bar=500 nm; n=nucleus.

Figure 6. Cytoplasm of plugs . a) Tegumented (white arrow) and non-tegumented (black arrow) capsids associated with keratin filaments (arrowheads) within cytoplasm of plugs; bar = 500 nm. b) Enveloped single capsids (arrows) among delicate fibrils and electron-dense globules within cytoplasm of plugs; bar = 400 nm. n=nucleus. c) Multiple capsids enveloped in electron dense material within cell cytoplasm

678 (arrow); bar = 200 nm. d) Low magnification of infected cell with swollen nucleus and vacuolated

679 cytoplasm; note electron dense material arrayed near cell membrane (arrows); bar = 4 µm.

Table 1: Log odds with 95% lower and upper confidence intervals, Chi square and p value results of Firth's logistic regression to evaluate contributions of tissue culture conditions to formation or maintenance of epidermal intranuclear inclusions in plugs.*

Parameter	Log odds	lower 0.95	upper 0.95	Chisq	p
Tumor surface area	0	-0.001	0	0.354	0.552
Days plugs submerged	0.106	-0.123	0.337	0.816	0.366
Days plugs at air-liquid interface	0.052	-0.011	0.119	2.583	0.108
No inducer	-0.088	-2.361	4.807	0.003	0.954
Sodium butyrate	1.039	-1.475	5.992	0.531	0.466
Dexamethasone	0.583	-4.784	5.959	0.073	0.787
Epinephrine	0.499	-4.94	5.96	0.05	0.823
Forskolin	-1.049	-4.225	4.028	0.31	0.578
Hexamethylenebisacetamide	-0.005	-5.332	5.322	0	0.998
Iododeoxyuridine	-0.991	-6.313	4.328	0.216	0.642
Tumor necrosis factor	-0.51	-5.817	4.793	0.059	0.808
Phorbol 12-myristate 13-acetate	1.24	-1.059	6.141	0.91	0.34
Trichostatin A	-0.289	-5.679	5.111	0.018	0.894

*No data for Valproic acid or 5-azacytidine because rafts for these plugs did not have intact epidermis to examine by histology.

Table 2. Log odds with 95% lower and upper confidence intervals, Chi square and p value results of Firth's logistic regression to evaluate tissue culture parameters for their contribution to formation of Eepidermal intranuclear inclusions in rafts.

Parameter	Log odds	lower 0.95	upper 0.95	Chisq	p
Keratinocyte (KC) passage number	0.21200	-0.14100	0.58000	1.46725	0.22578
Collagen concentration (mg/ml)	-0.69300	-3.24000	0.58200	1.06324	0.30248
Fibroblasts/ml collagen	0.00000	-0.00001	0.00001	0.00257	0.95955
Hours collagen raft allowed to shrink	0.02860	-0.01560	0.07730	1.72554	0.18898
Day collagen raft detached from well	-0.02380	-0.33500	0.34700	0.02203	0.88201
Shrinking temperature of collagen raft	0.05020	-0.90500	0.59400	0.02569	0.87265
Day collagen raft incubated prior to addition of KC	0.84000	0.03290	1.95000	4.18706	**0.04073**
KC/surface area of raft	-0.00005	-0.00021	0.00002	1.94728	0.16288
Days rafts Submerged	0.48600	-0.03890	1.27000	3.32585	0.06820
Days rafts at air-liquid interface	0.13800	-0.25500	0.80200	0.50773	0.47612

Table 3. Mean, standard deviation (SD), number (n), and range of diameters (nm) of viral capsids within nucleus and cytoplasm of cultured plugs and rafts categorized by capsid type. TLC indicates capsids were too few to count or summed to less than or equal to three. Eight, 19, and 11 cells were enumerated for plug cytoplasm, plug nuclei, and raft nuclei, respectively.

	Plugs			Rafts	
	Mean ± SD	Range		Mean ± SD	Range
Capsid type	Nucleus				
Clear	100 ± 7 (61)	86 - 118		111 ± 8 (51)	89 - 128
Concentric	101 ± 8 (63)	86 - 124		109 ± 9 (102)	87 - 131
Stippled	100 ± 6 (60)	86 - 117		0	0
Targetoid	100 ± 7 (18)	88 - 111		130 ± 20 (11)	101 - 177
Solid	101 ± 3 (4)	98 - 103		94 ± 9 (14)	78 - 105
	Cytoplasm				
Tegumented	214 ± 20 (24)	175 - 270		TLC	TLC
Non-tegumented	217 ± 32 (64)	165 - 306		TLC	TLC
Enveloped	238 ± 41 (11)	190 - 306		TLC	TLC

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